# Formulation, Characterization and Bioefficacy of Alum based Nanoemulsions for controlling *Aedes aegypti*

A

Synopsis

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## Introduction

Mosquitoes are the most serious threat to society since the dawn of the civilizations, compared to any other disease. Mosquito borne diseases contribute to approximately 17% of the world's total diseases, causing almost 5,00,000 deaths annually (WHO, 2017), which are higher than deaths caused by any other taxon of animals. Approximately 3,500 different species of mosquito have been reported so far in the entire world, out of which India houses 404 species. Among these three major genera *viz.*, *Anopheles, Culex* and *Aedes* spread several protozoan, nematodes and viral pathogens. *Aedes* is the major species, primarily responsible for transmitting dengue and chikungunya virus and is also a vector of flavivirus and recently zika virus (Gubler, 1998). Dengue is endemic in 100 countries (WHO, 2016) including India. Earlier incidences of dengue cases were 100 million annually (CDC, 2009). Which are now increasing dramatically with an average of 390 million cases annually in the entire globe, putting one-third population of the world at risk (WHO, 2019). Its speed of spreading is high with 50 million new infections annually in the world (Brady *et al.*, 2012). The year 2012 was considered as the year of highest dengue burden in the world, with high potential of epidemic. Adding complexity in dengue transmission understanding, recently a new and fifth serotype of dengue has been discovered, but it is not prevalent in India (Mustafa *et al.*, 2015). The year 2016 accounted for approximately 1,00,000 cases of dengue in India (Ganeshkumar *et al.*, 2018). Such high dengue burden is draining the economy of the country and is putting the stress on the health systems. Information of the dengue disease burden, its geographical distribution and its prevalence is necessary for its control, but very scarce and inadequate data is available regarding the status of dengue vectors prevalence in India. Only a few studies have reported the estimates of this disease status and severity (Amarsinghe *et al.*, 2014).

The rigorousness and number of the diseases caused by *Aedes* species are high and the figures are quite alarming, therefore their control is the need of the hour. Vector control has been an important part of the global strategy to manage this vector and insecticide use is the vital component of dengue control programmes. The last few decades have evidenced the immense use of insecticides, accounting

nearly 70,000 tonnes of insecticides to combat the problem (Jovanovic *et al.,* 2011). Due to the high and continuous use of insecticides, the problem of resistance has emerged.

Nanotechnology, the technology of the 21st century has revolutionized the world with various applications because of its nano size products. Nanotechnology has found its applications in gene delivery, drug delivery, parasites, pest and vector management (Priya, 2015). The green approach of the nanotechnology for vector management has caught the attention because of its safe, cheap, non-toxic nature. Various products of nanotechnology include nanorods, nanofibres, nano-optics, nanomedicines and plenty others with tremendous potentials. Nanoemulsions are one of the most advanced and successful products used in drug delivery of therapeutic agents (Savardekar and Bajaj, 2016). Several types of nanoemulsions have been formulated by various researchers utilizing the medical properties of different oils. Various non- essential and essentials oils used in nanoemulsions preparation includes *viz.,* neem oil, rosewood oil, geranium oil, eucalyptus oil, castor, coconut oil, primrose oil, linseed oil, olive oil and various other (Nikam *et al.,* 2018).

Mosquito's survival depends on its nutritional reserves *viz.,* protein, lipid and glycogen, which they acquire by feeding in their lifetime, both as an adult and as larvae, and under stress condition like competition and unavailability of food. They utilize the reserves for their survival and reproduction to produce progenies (Briegel, 1990). Lipids are required for long-term maintenance, flight and mating (Ng' habi *et al.,* 2005). This directly indicates that lipid depletion at larval stage may hinder reproductive and survivorship capabilities, resulting into affected vectorial capacity (Chotiwan *et al.,* 2018). This provides an insight into development of such formulations and larvicidal agents, which will target and diminish the stored lipids in the body of larvae. This will pave a way of controlling the dengue vector and in reducing the disease burden, which is responsible for millions of deaths every year with stunt social and economic development of countries around the globe.

Alum is a traditionally known double salt used in Indian and Chinese medicine. It is also known with the name phitkiri, saurashtri. In the Chinese medicine system, it is popular as ming fan. It has found its uses as astringent, flocculent, mordant, in cosmetics, food preparation and various other fields. It is cheap and easily available compound. Depending upon the monovalent cation, alum can be classified

as potassium alum, sodium alum and aluminium alum, out of which potash alum has been explored in the past years for its pest management and insecticide applications (Preet and Seema, 2010; Sathyan *et al.*, 2015; Elham *et al.*, 2016).

From the above lines, it can be understood that the nanoemulsion in combination with alum, could work as potential nanolarvicides which possibly have a detrimental effect on the stored energy reserves of mosquito larvae. Hitherto, no study has been documented regarding impact and assessment of nanoemulsions of alum and essential oils, and their impact on reserved energy source *i.e.*, lipids of *Aedes aegypti*. Therefore, keeping this in mind and to bridge the gap, the effect of nanoemulsions prepared using essential oils and alum combination will be evaluated on dengue vector.

**Review of literature**

Mosquitoes are one of the most primitive insects found on earth. Their history dates back to about 80-90 million years ago, with a total number of 3,500 species. They are considered as the most deadly organisms ever found on earth. *Aedes* is medically one of the most important species of mosquito. It is responsible for transmitting WHO's (World Health Organization) most concerned disease dengue. According to the report of (WHO, 1997), dengue is the most prevalent mosquito borne diseases and is endemic in 100 countries including India. It is caused by the dengue virus (DENV, 1–4 serotypes), which is one of the most critical arboviruses found in tropical and subtropical regions of the world. The World Health Organization (WHO, 2009) developed guidelines for dengue and its fever classification. As per the report of the National Centre for Disease Control (CDC, 2009), dengue has various manifestations, ranging from dengue fever to dengue hemorrhagic fever (DHF) and dengue shock syndrome.

Epidemiological and ecological status studies are important for proper vector management. Various studies and surveys have been done by the researchers in past years, revealing the status of *Aedes* species prevalence in India. Studies done by Ramakrishnan *et al.* (1964) reveals that almost all the states and union territories of India have a history of *Aedes* infection and diseases like dengue and chikungunya. Studies done on dengue vector's prevalence by Pandya (1982) indicated that the first incidence of dengue fever recorded in India was in Kolkata in the year 1963, slowly crossing its boundaries, it got spread in the entire country, namely Orissa, Arunachal Pradesh and Mizoram, where dengue was historically non-existent. According to the studies of Chaturvedi and Nagar (2008) year 2006, was a black year in India, recording 1,023 cases and 423 deaths due to dengue in the capital city Delhi only. Reports by Tripathi *et al.* (2008) reports that Uttar Pradesh has also experienced major dengue outbreaks and the cases are reported both from urban and rural areas. *Aedes albopictus*, which was earlier considered as a forest species have been reported in human vicinity. Kumari *et al*. (2011) have reported incidences of *Ae*. *albopictus* in Delhi for the first time. Surveillance studies conducted by Gupta *et al.* (2014) in the city Agra, reported the presence of both the major species of *Aedes* genus *i.e., Aedes aegypti* and *Ae. albopictus*. But this study indicated the higher prevalence of *Aedes aegypti*

compared to *Ae. albopictus* in Agra city. The studies were conducted from the year 2009-2012. It was also evident from the following study that the population of *Ae. albopictus* is at a continuous increase from last years. The study also revealed various breeding habitats of the *Aedes* sp. Coolers were found to be the major breeding sites, apart from this, unused tires, cement tanks, garden pots and pits were also the preferred habitats of *Aedes* sp. A pilot study was conducted from March 2019- October 2019, which indicated the higher prevalence of *Aedes aegypti* compared to *Ae. albopictus* in Agra city. *Aedes aegypti* larvae were collected from various breeding sites including, cemented tanks, coolers, flower pots, unused tyres etc.

The reason behind such high prevalence of mosquitoes all around the world could be assigned to their extraordinary abilities to survive in adverse environments. Their survival in harsh conditions is due to the reserved energy sources in their bodies. The fat bodies, distributed throughout the insect body are of great importance in the life of insects. They are found underneath the tegument, surrounding gut and reproductive organs. Lipid maintains the energy levels and helps insect meet their energy demands. Lipids are important as they are responsible for the growth, reproduction, flight and metamorphosis in mosquitoes and also prevent their bodies from desiccation. Thus, targeting these energy reserves using insecticide or formulations may help in getting rid of these disease vectors. Researchers around the globe, have found out the detrimental effects of chemical insecticides on lipid content and compositions. Sharma *et al.* (2011) have evaluated the effect of *Azadirachta indica* and *Artemisia annua* ether extract on lipid content depletion. Sushchik *et al.* (2012) have done studies on the comparison of the larval and adult lipid composition of different mosquito species. Sosa *et al.* (2014) have documented the study on insect lipid profile extracted via different methods. Sneha and Preet (2016) have recently evaluated the effects of sublethal conventional and biorational larvicides on lipid content depletion.

Alum is a chemical compound with name hydrated double sulphate salt of aluminium with chemical formula $KAl(SO_4)_2.12H_2O$. It is also called as Potassium alum or potash alum. It is an EPA (Environmental protection agency, 1970) approved pesticide (FIFRA, 1996). It has the potential to control and manage both pest and mosquitoes.

Studies done by Preet and Seema (2010) reported the high potential of alum in controlling malarial vector. Another study done by Preet and Sneha (2011) indicates the potential of potash alum in affecting the tegument of dengue vector, working as a contact poison for these soft bodied creatures. Sathyan *et al.* (2015) reported the use of alum as an insecticide to manage the agricultural pest. Salama (2015) explored the potential of alum as an insecticide in controlling *Periplaneta americana*. It is an eco-friendly, economic, readily available and potential agent to control the pest and mosquitoes and thus can also be explored for control of other mosquito species. It can also be used in combination with nanotechnology to get extraordinary results in the vector control field.

As the number of dengue, chikungunya and other *Aedes* borne diseases are accelerating, there arouses a perpetual need to control of vector and subsequent spread of disease. Nanotechnology comes out here as a potent solution, because of the failure of other control programmes due to problem of resistance. Nanoemulsions are the most advanced form of nanoparticles systems. They are submicron-sized emulsions containing oil and water, stabilized by a surfactant, which are a good agent for therapeutic delivery due to their nano size. Nanoemulsion size generally ranges between 10-100 nm, smaller size and increased surface area enhance their effectiveness. Savardekar and Bajaj (2016) reported the unique plausible characteristics of nanoemulsions which have caught the attention of researchers because of their robust stability, tunable rheology and targeted drug delivery. Gupta *et al.* (2016) reported the importance of plants as the source of essential oils, which contains bioactive compounds like terpenes, phenols, alcohol, ether, aldehydes, terpenoids, ketones and various others. Essential oils are the volatile oils obtained from plants and their secondary metabolites. These volatile oil are obtained from various plant parts *viz.*, leaves, twigs, bud, seeds, bark, stem, fruit, roots, wood, flowers and herbs. Most of the essential oils are approved by FDA (Food and Drug Administration) and put in the category of GRAS (Generally Regarded as Safe). Essential oils are prevalent in pharmacies and they are well known for their role as antifungal, antibacterial, antioxidant, antidandruff, spasmodic and as an insect repellent (Nirmala and Nagarajan, 2017). Aromatic and lipophilic bioactive compounds found in essential oils pose disadvantages like poor aqueous solubility, low stability, limited administration routes and strong organoleptic characteristics; therefore they need

a nanocarrier for their controlled release and enhancing their effectiveness. Various plant essential oils are also considered as an important source of natural insecticide. They are safe, selective in action and non-toxic in nature. Extraordinary properties of essential oils and nano dimensions of nanoemulsions are combined these days to produce potential larvicides for controlling mosquito borne diseases. Balasubramani *et al.* (2017) have tested the nanoemulsion of *Vitex negundo* against *Aedes aegypti*. Jesus *et al*. (2017) have recently evaluated the nanoemulsion of *Carapa guianensis* against *Aedes aegypti.* Nanoemulsions also exhibit antioxidant, repellant, anticancer and antibacterial properties (Nirmala and Nagarajan, 2017). Essential oils of *Azadirachta indica* has been checked against coleopterans (Choupanian and Omar, 2018). Balasubramani *et al.* (2018) have evaluated the activity of essential oil of *Ocimum basilicum* against *Culex* species. Sogan *et al.* (2018) studied the potential of castor oil nanoemulsion against *Anopheles* species. Recent studies done by Ferreira *et al.* (2019) showed the use of *Lippia alba* nanoemulsions for their larvicidal activity against chikungunya vector.

Only a few essential oils have been evaluated for their potential as alarvicide. And a little data is available on nanoemulsions larvicidal activity for *Aedes aegypti*. Other essential oils are also expected to have larvicidal activity, which needs to be explored for controlling dengue and chikungunya vector.

Accordingly, this study will deal with the synthesis of nanoemulsions using essentials oils of plants and potash alum and their larvicidal impact on dengue vector. The study will continue further by evaluating the impact of the synthesized nanoemulsions, on the stored energy reserve *i.e.,* the lipid content of *Aedes aegypti* larvae. Risk assessment of synthesized alum based nanoemulsions will be done to assess the safety of above said nanoemulsions. Concludingly, this study will help develop an efficient nanoproduct for reduction of dengue burden.

## Objectives

The study will include following objectives:

1. Formulation and characterization of alum based nanoemulsions.
2. Bioefficacy of alum based nanoemulsions and their sublethal effects on lipid profile of *Aedes aegypti*.
3. Risk assessment study of potent nanoemulsion on Human embryonic kidney 293 (HEK 293) cell lines.

## Materials and methods

### Materials

**Experimental animal: *Aedes aegypti*** (Linn, 1762)

**Characteristics and Phylogeny**

*Aedes aegypti* is a blackish, dark coloured mosquito with white lyre shaped markings and banded legs, measuring approximately 4-7 mm in length. They are visually distinctive because of the alternate black and white markings on their body as well as legs. Females can be distinguished from males in possessing small palps stripped with silver or white scale. Larvae of *Aedes aegypti* are bottom dwellers and perform a wriggling 'S' shaped movement. They prefer to stay in dark and when disturbed prefer to go deeper. They can be identified with the help of short respiratory siphon and darker colour. This species was first named by Linnaeus, in 1762.

**Distribution and Habitat**

This species was first originated in Africa, but now they are found all around the globe. It is found in 23 countries, with the highest population in Northern Brazil and Southeast Asia, including India. It is found in areas lacking the piped water system. They are found in natural or artificial containers which are left undisturbed for long and in close proximity of human inhabitation.

**Medical importance**

*Aedes aegypti*, found in tropical and subtropical countries, is considered primarily the vector of dengue fever, chikungunya and yellow fever. It is also responsible for the transmission of some economically less important diseases like west nile virus, eastern encephalitis, zika virus and notably many others.

**Experimental materials**

**Essential oils**

Terpenoid, thymol and citral rich essential oils will be purchased from Sigma Aldrich and will be used for the synthesis of nanoemulsions.

*Azadirachta indica* (Neem) is an evergreen Indian tree belonging to the family Meliaceae. It contains various bioactive compounds, and the major one is triterpenoid. Azadirachtin and Nimbin are two important triterpenoids, which are responsible for various bioactive properties of Neem. Neem oil is extracted from neem fruit seed kernels. The antifeedant, antibacterial, antibacterial, antifungal repellency, pesticide, insect growth inhibitory and many other activities of neem oil has been reported so far (Rajendran, 2012; Campos *et al.*, 2016; Choupanian and Omar 2018).

*Trachyspermum ammi*, commonly called as Ajwain or Ajowan. Caraway is the annual herb of family Apiaceae. Ajwain oil is extracted by hydrodistillation of Ajwain seeds. Ajwain oil is rich in terpenoids, which is responsible for its various medicinal uses in ayurveda.it is also known for it's nematicidal, anthelmintic and antifilarial activities (Bairwa *et al.*, 2012; Chahal *et al.*, 2017).

*Thymus vulgaris* is the flowering plant of family Lamiaceae, native to southern Europe. It has both culinary and medicinal importance. It contains monoterpenoids and glycosides. Its main composition is thymol and carvacrol. Its flowering aerial parts and leaves are used for oil extraction. It is famous as folk medicine since ages and is found to have antimicrobial, antioxidant, anthelmintic, antimicrobial and many other properties (Xu *et al.*, 2008; Kumari *et al.*, 2018).

*Cymbopogon citratus* is commonly called as West Indian lemongrass. It is a tropical plant native to Southeast Asia. It contains citronellol and citral as its main bioactive compounds. Its leaves are used for oil extraction. Lemongrass oil is found to have antioxidant, anti-inflammatory and antimicrobial, anti-fungal properties (Sharma *et al.*, 2018; Gago *et al.*, 2019).

Except *Trachyspermum ammi*, above oils have been utilized in synthesizing nanoemulsions and have been evaluated for their various biological properties *viz.*, insecticidal, anti-parasitic, antimicrobial, antioxidant and many other important activities. But these have not been explored for their larvicidal activities, specifically against *Aedes aegypti*. Keeping this in mind and to bridge the gap in the studies regarding their biological activities, the above discussed oils has been chosen.

**Surfactant : Tween 80**

Polyoxyethylene (20) sorbitan monooleate is non-ionic surfactant and emulsifier. It is also known as Polysorbate 80. It is also sold in the market under the name Tween 80, montanox 80, alkest TW 80 and PS80. It is derived from polyethoxylated sorbitan and oleic acid. The hydrophilic groups in this compound are polyethers, also called as polyoxyethylene groups, the polymers of ethylene oxide. The surfactant is used in nanoemulsion synthesis to reduce the interfacial tension between oil and aqueous layers and to yield stable emulsion.

**Potash Alum**

Alum is traditionally known double salt in Indian ayurvedic medicine system with chemical formula $KAl(SO_4)_2.12H_2O$. It is also called as Potassium alum or potash alum. It is an EPA (Environmental protection agency) approved pesticide. It has the potential to control and manage both pest and mosquitoes. Consequently, alum will be used in the synthesis of nanoemulsions, to make them more effective and potent against *Aedes aegypti* larvae. Using alum in nanoemulsion preparation is hypothesized to increase the larvicidal potential of nanoemulsions, making it a more noxious larvicide.

**Human embryonic kidney 293 (HEK 293) Cell lines**

Human embryonic kidney 293 (HEK 293) cell lines will be used for the risk assessment study. HEK cell lines are derived from human embryonic kidney and have a role in filtering toxicity from the body. These cell lines have been used by several researchers for assessing the cytotoxicity of various novel nanoformulations (Sharma *et al.,* 2018). Therefore, for assessing the toxicity of the synthesized nanoemulsions these cell lines have been chosen.

**Methods**

**Formulation of alum based nanoemulsions**

For the preparation of alum based nanoemulsion, crude alum will be procured from the local dealer while standard alum and essential oils will be purchased from Sigma Aldrich. Before using essential oils for the synthesis of nanoemulsions, they will be characterized for their phytochemical screening of

compounds using Gas chromatography-Mass Spectrometry (GC-MS) using the protocol of Nuchuchua *et al.* (2009). GC-MS will be done at AIRF, JNU, New Delhi. For nanoemulsion synthesis, the protocol of Sugumar *et al.* (2015) will be followed. Nanoemulsions will be synthesized, with and without alum. Briefly, different ratios of essential oils, surfactant (1:0.5, 1:1, 1:1.5, 1:2) and alum (0.1%, 1%, 10%) will be mixed with milli Q water to get a coarse emulsion with the help of magnetic stirrer. Later, this coarse emulsion will be subjected to sonication using a sonicator, at a particular frequency for multiple cycles. Sonication will produce strong disruptive forces and reduce the droplet size of emulsions, converting it from coarse emulsion to nanoemulsion.

**Stability testing and characterization**

After the synthesis, the nanoemulsions will be tested for their stability. For this, the synthesized nanoemulsions will be centrifuged at 3,500 rpm to ensure their stability. After this, six cycles of heating (45°C), cooling (4°C) will be done for 48 hrs. each. The final stage of stability will be checked by Freeze-thaw cycles, the nanoemulsions will be subjected to rapid cooling (-21°C) and heating (25°C) alternatively for 48 hr. each. This cycle will be repeated 3 times to ensure the stability of synthesized nanoemulsions.

After this, the nanoemulsions will be characterized, by making appropriate films or solutions using milli Q water to avoid multiple scattering effects. For confirming the droplet size, polydispersity index (PDI) and zeta potential, the Dynamic Light Scattering (DLS) studies will be done. DLS will be done in DEI, Agra. For studying the morphology of nanoemulsions, Transmission Electron Microscopy (TEM) and Fourier Transform InfraRed (FTIR) studies will be done to find out the structural details and functional groups in nanoemulsions respectively. TEM will be done in AIIMS, New Delhi while FTIR studies will be done at AIRF, JNU, New Delhi.

**Larval collection**

The present study will be conducted in Agra city (27°10N, 78°05E), situated in the extreme south-west corner of Uttar Pradesh. Agra is situated in the semi-arid zone of Northern India. Larvae will be collected from various breeding sites. They will be collected using standard dipping procedure.

**Larvicidal bioefficacy study**

Larvicidal bioefficacy will be carried out following the standard guidelines of the World Health Organization (WHO, 2005). 250 ml of deionized water will be used for conducting larvicidal tests. For this, 25 late third instar larvae will be used against every concentration. 5 replicates of each set will be placed. Both positive and negative control will also be placed along with the test concentrations using permethrin, surfactant and water.

The stock of larvicides (synthesized nanoemulsions) will be prepared, and from those, the working concentrations will be prepared. In starting a broad range of concentrations will be tested, later on, $LC_{50}$ and other sublethal concentration will be evaluated, and sublethal concentrations will be prepared accordingly for further lipid analysis. The percent mortality will be recorded at 24, 48 and 72 hours. Larvicidal susceptibility will be expressed in terms of $LC_{50}$ and $LC_{90}$ using probit analysis (Finney, 1971). Control mortality will be corrected using the abbot formula (Abbott, 1925) as follows:

Abbott's formula =T-C/100-C*100

Where, T= % mortality in treatment, C= % mortality in control

**Sublethal effect of nanoformulation on lipid profile**

Based on larvicidal bioefficacy study, sublethal concentrations will be calculated and *Aedes aegypti* larvae will be exposed to sublethal concentrations ($LC_{10,}$ $LC_{25}$ and $LC_{50}$) for studying their impact on lipid profile as follows:

**Lipid extraction**

Lipid extraction will be done following the protocol of Van Handel (1985) with slight modifications. Briefly, 5 larvae from each treatment group will be transferred to eppendorf and crushed using sodium sulphate. For preparing homogenate, 1.6 ml of chloroform: methanol (1:1) will be mixed to the above homogenate and the mixture will be centrifuged at 3,000 rpm for 1 minute. The supernatant will be transferred into another fresh tube and pellet will be discarded. To this supernatant, 1.6 ml of water

will be added and centrifuged again at 3,000 rpm for1 minute, the upper layer will be discarded, the pellet containing lipid will be stored in -20$^0$C for further analysis.

**Quantitative total lipid content analysis**

For quantitative lipid analysis, the solvent will be evaporated completely in the heating block, and sulphuric acid will be added. After heating for a few minutes, 5 ml of vanillin-phosphoric reagent will be added. Samples will be cooled at room temperature, to develop pinkish-red colour. Absorbance will be read at 625 nm. The standard graph will be prepared for lipid concentration using vegetable oil. Using the standard graph, the total lipid content will be calculated.

Lipid depletion index in treated larvae will also be calculated (Van Handel, 1985).

LDI =C-T/C+T X 100

Where, C= absorbance of the control sample, T= absorbance of the treated sample

**Qualitative lipid analysis**

For this, the protocol of Sushchik *et al.* (2012) will be followed. Briefly, lipid will be extracted following the above given steps of lipid extraction. The extracted lipid will be fractionated by thin layer chromatography on silica gel G with system hexane–diethyl ether–acetic acid (85:15:1, v/v). The lipid spots on plates will be identified according to reference compound position.

**Risk assessment study**

Human embryonic kidney 293 (HEK 293) cell line will be used for the risk assessment study. MTT (3-(4, 5-Dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide) assay will be done to check the toxicity of potent alum based nanoemulsions on mammalian cell lines, following the procedure of Mossman (1983). MTT assay will be performed to assess the cytotoxicity. MTT is a yellow dye, which is reduced into purple formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells. Human embryonic kidney 293 (HEK 293) cells will be cultured

in 96-well microtitre plates using DMEM (Dulbecco's modified eagle's medium) along with 10% FBS and 1% penicillin streptomycin as a culture media followed by overnight incubation at 37°C and will be treated with varying conc. of different alum based nanoemulsions for 24 and 48 hrs. At the end of the treatment period, to each well, 10 µL of MTT will be added. After the addition of MTT, the plates will be incubated for 3 hrs in a dark chamber and supernatant from each well will be aspirated. Then, 100 µL of DMSO (Dimethyl sulfoxide) will be added to dissolve the formazan crystals. The absorbance will be read at 595 nm using the microplate reader. Percent cell viability will be calculated as follows:

Percent cell viability= (Absorbance of treated cells/Absorbance of the untreated cell) *100.

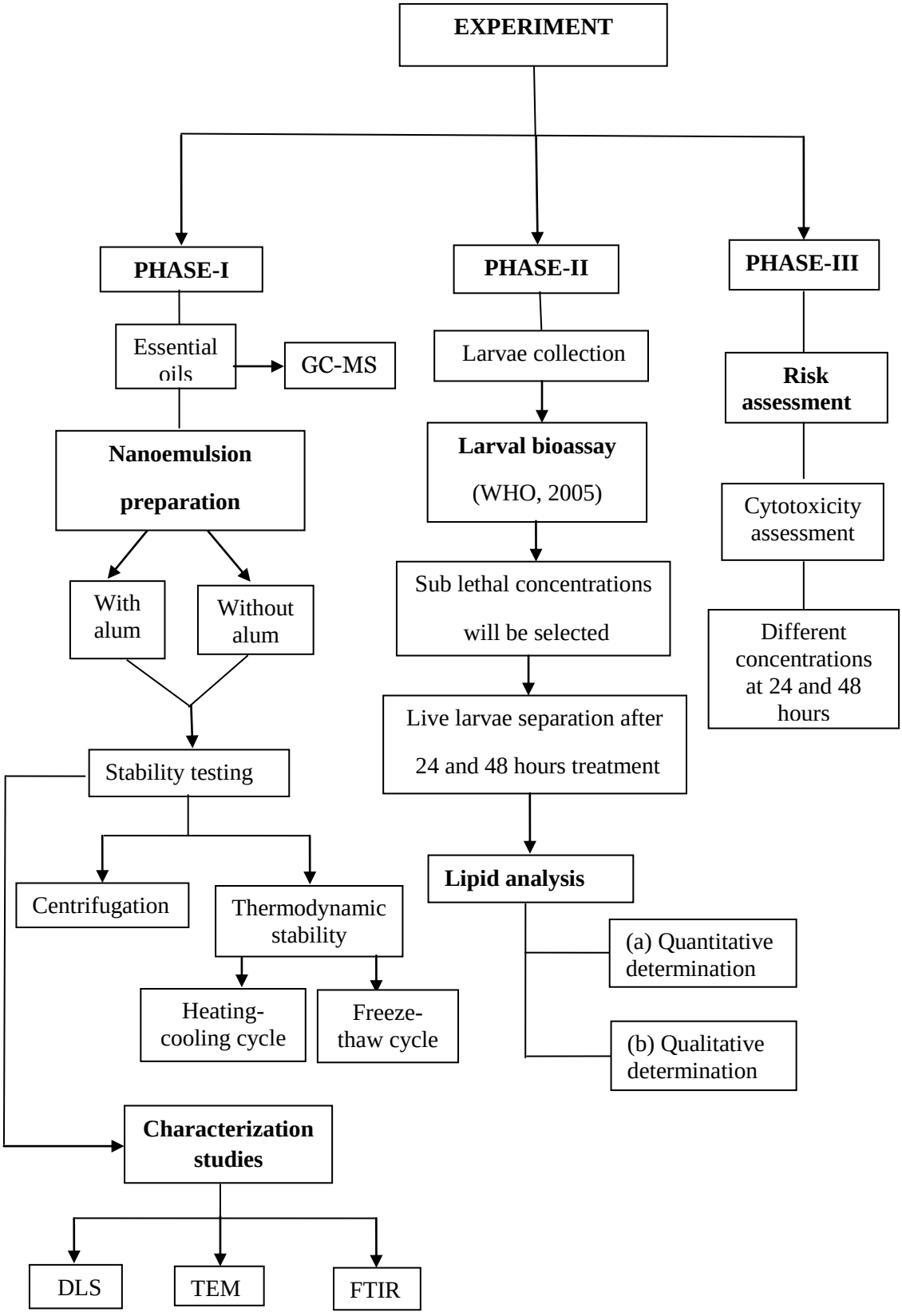
**Statistical analysis**

All the results will be analyzed statistically. The data will be represented in mean ± standard deviation. The quantitative estimation of lipid content will be done with the help of the regression equation. ANOVA will be used for mean difference analysis between treated and control groups. SPSS and Graph pad prism will be used for statistical analysis and processing and analyzing the data.

**Preliminary work done**

- Comprehensive literature survey has been done.
- A pilot study has been performed for the selection of alum ratios with essential oil for formulating nanoemulsions.
- Optimization of protocols for nanoemulsions synthesis.

**Experimental design**



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